STABLE SPECTROPHOTOMETRY
OF SMALL DENSITY CHANGES

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Stable Spectrophotometry of Small Density Changes*

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Measurement and control circuits for recording small density changes by use of several photo-tube circuits are given. The minimum drift rates obtained are $1 \times 10^{-6}$ part per minute with a differential photo-tube circuit, $3 \times 10^{-5}$ part per minute with a single photo-tube circuit, and $4 \times 10^{-4}$ part per minute with a photo-multiplier circuit. The differential and single photo-tube circuits have been used to measure density increments of roughly $2 \times 10^{-4}$ and $1 \times 10^{-3}$ with an accuracy of about 5 percent. These spectrophotometers are employed in studies of the kinetics of unstable intermediates in enzyme action. Although slow drift has been rendered negligible by suitable control circuits and stable amplifiers, the theoretical limit of performance is not achieved by this apparatus.

INTRODUCTION

A PREVIOUS paper1 described control and measurement circuits based upon direct-coupled amplifiers for studying rapid chemical kinetics. The reader is referred to that paper for a description of the purpose and functions of the photo-tubes and filters. In this paper, an improved apparatus is described which employs mechanical-switch modulators and demodulators for both measurement and control circuits. These circuits are about fifteen times as sensitive as those previously described and are more stable and reliable. In addition, a comparative study of various photo-cell and photo-multiplier circuits is given.

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The use of packaged amplifiers and mechanical-switch modulators and demodulators for low level d.c. measurements has been given elsewhere.2 The particular input and output circuits used here are given in the following discussion.

1. DIFFERENTIAL PHOTO-CELL CIRCUIT

1.1 Design

After a few preliminary experiments on direct modulation of the photo-tube anode voltage using a signal-and-carrier-balanced configuration,3 it became apparent that the control of the carrier waveform and the balancing of the stray capacitances were more difficult than in the case of the mechanical-switch modulation. In addition, an upper limit to the carrier frequency of 100 c.p.s. was satisfactory and the reduction of flicker noise obtainable by operating at a high
carrier frequency was not required since shot noise in the photo-current usually predominates. The final input circuit design is indicated in Fig. 1 where a pair of photo-tubes is used to measure the shift of an absorption band from 405 to 420 μm, the peaks of the filters being at 390 and 425 μm (see reference 1). The photocurrent of the two photo-tubes is equalized by an initial mechanical adjustment. The input to the a.c. amplifier (designated by the triangle) is alternately switched at 60 c.p.s. from one phototube to the other by the 6.3 v excitation of the magnetic field of the Brown Converter. The carrier pick-up is reduced by removing the converter from its socket and by shielding the 6.3 v carefully, and by adjusting the centertap of the carrier voltage. Equal photo-cell load resistors are used to avoid drifts caused by changes of amplifier grid current.

A second channel measures the light absorption at a third wave-length, in this case, at roughly 580 μm, and a dummy photo-tube is employed to equalize the pick-up. An adjustable bias is provided from the 50-ohm potentiometer to equalize the voltage at the contacts of the switch. The outputs of both modulators are connected to amplifiers having a gain of 1000 and a few microvolts noise level over a 1 kc/sec band width.

The output of both these preamplifiers is further amplified and demodulated as shown in Fig. 2. A calibrated attenuator is interposed between the first and second amplifier units, and over-all sensitivities varying from 1.5 to 25 μv per division of the output meter are available. The second amplifier unit is identical to the first but has no requirement for low noise level. Its output is connected to a stepdown transformer with pushpull output for operating the mechanical-switch demodulator. The duty ratio of the demodulator is adjusted to 0.9 that of the modulator to avoid sensitivity to small changes of the duty ratio of either switch and errors due to inadequate band width of the a.c. amplifiers. A four-section RC-filter is used to control the rise time (10-90 percent) of the output voltage and thereby to obtain improved signal-to-noise ratio when a slow change is being measured.

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4 For details of these devices, the reader is referred to the Brown Instrument Company, Philadelphia or to Radiation Laboratory series Vol. 17, Section 13.6.
The signal-to-noise ratio is, in fact, determined by the band width of the network following the phase-sensitive demodulator even though the band width at the carrier frequency is roughly 1kc/sec. The theoretical noise band width of a multiple-section network of this type is equal to its 3-db band width, while the rise time is but one-third the reciprocal of the 3-db band width or $2.1RC$, since the latter is equal to $1/2\pi RC$.

There is, however, only a small loss of signal-to-noise ratio when a single-section filter is used. Filters having rise times varying from 0.5 to 0.005 sec. are provided. In the latter case, however, the delay caused by the switching frequency ($1/120$ sec.) is appreciable, and considerable ripple is present. The level at the output of the filters is 50 mv per division and is satisfactory for amplification by well-stabilized direct-coupled circuits. A low level output is desirable here to avoid saturation of the output stage of the a.c. amplifier by noise voltages. The quiescent level of the output is adjustable between 4 and 8 volts by the 1K potentiometer. The oscillograph driver circuit (Fig. 3) and its characteristics have been discussed elsewhere. There are six recording channels; all are recorded on a 6-element oscillograph, and two are also recorded on Esterline Angus recorders for slow changes of light intensity and for calibration.

A record of the performance of the modulator, demodulator, and recorder elements is given in Fig. 4 where the output is recorded at full gain for a period of 5 hours with the photo-tubes disconnected. The sensitivity is $1.8 \mu v$/division, and the drift rate over the 5-hour period is roughly $6 \times 10^{-9}$ volt per minute, a figure which is far superior to that obtained from well-stabilized d.c. amplifiers (several microvolts per minute) by a factor of about 1000.

The over-all linearity of the system is better than 2.0 percent, and the range is limited by saturation of the cathode-follower of the second a.c. amplifier (provided the diodes of the oscillograph driver are inoperative). Direct feedback over the entire loop would give greater linearity and gain stability.

A highly stable amplifier is, however, useless for sensitive spectrophotometry unless the light intensity is also stabilized. One type of regulator for a tungsten lamp is shown in Fig. 5. Here two types of control are available; one is a voltage control operating from a portion of the lamp voltage and using a d.c. amplifier and a 6AS7G control tube shunted by a small resistor. This circuit is adequate for many measurements, especially if the 110v d.c. line is well stabilized, i.e., battery operated. Considerably greater gain at low frequencies is afforded by a photo-tube-
FIG. 5. Light-Intensity Regulator. Rapid fluctuations are reduced by the low gain wide-band d.c. amplifier voltage controller, while slow fluctuations are reduced by the narrow-band high gain photoelectric controller which is responsive to the temperature of the lamp.

ccontrol circuit also shown in Fig. 5. Two phototubes, operating in a differential circuit, measure the preponderance of blue over red light as the filament temperature increases. This differential signal operates a modulation-demodulation system similar to that shown in Figs. 1, 2, and 3. In this case, the band width is made very narrow in order to obtain stable operation; a rise time of 0.8 sec. is usually used (1M X 4 mf). Since the filter output is at a high resistance, a spare driver unit (any impedance changer would do as well) is used to add the photo-tube control to the voltage control. In this way the rapid fluctuations (to which the measuring circuits are not very sensitive) are removed by a low gain wide-band control circuit, and the slow fluctuations are removed by the high gain narrow-band photo-tube controller. Since the photoelectric control circuit must have a very slow response speed when used at very high gain, it is desirable to increase the gain of the wide-band voltage control. This is readily accomplished by inserting another amplifier unit in the d.c. loop. Such a feed-back loop is easily stabilized by restricting the band width at one point, preferably at the input of the d.c. amplifier shown in Fig. 5.

One objection to the differential photo-tube input is its low gain; the differential intensity does not change rapidly with lamp temperature. A higher gain input circuit employing only one photo-tube (with a blue filter) operates equally satisfactorily. Alternatively, more rapid response is obtained by using lamp voltage as a control signal in the narrow band circuit.

1.2 Performance

Some performance data of the circuits of Figs. 1, 2, 3, and 5 are shown in Figs. 6–8. Figure 6 shows the drift of the output with a sensitivity of 1.2 μV/div and a photo-current in each phototube of 0.02 μA or a signal of 2 X 30 mV. The drift...
rate, due nearly entirely to light-intensity fluctuations, is roughly 3 μv/min. over a 5-minute interval or a drift of 50×10⁻⁶ part per minute. The noise, due largely to more rapid light-intensity fluctuations, is roughly 3 μv. Both shot and thermal agitation noise are less than this over the Esterline-Angus meter band width (∼1/₅ c.p.s.).

The performance over a longer time interval (11 hours) at one-fifth the gain (6 μv/div.) is shown in Fig. 7. Here the rate is somewhat less, 0.04 μv/min. or 0.7×10⁻⁶ part/min., over the last 8 hours.

Figures 8a and 8b show the performances of the apparatus in measuring a small change of absorption due to the reaction of the hematin enzyme peroxidase with hydrogen cyanide. With a circular tube of 1-mm bore and a rapid-flow apparatus, the formation of the enzyme-inhibitor compound is recorded in Fig. 8a on an Esterline-Angus recorder and a mirror oscillograph in Fig. 8b. The final enzyme concentration was 1×10⁻⁸M Fe/L, and the formation of its cyanide compound gives a spectral shift at 400 μm corresponding to a density change of 1.9×10⁻⁴ provided a rectangular tube of 1-mm depth is used. Since a circular tube was used the effective trough depth was less than 1 mm, and a smaller change of light intensity occurred. Since the sensitivity was 1.2 μv/division, the signal was 16 μv or 2.6×10⁻⁴ of the effectively 60-mv signal. The noise level of Fig. 8b is roughly 2 μv, and it may be compared with a calculated 0.6 μv of shot noise (∼7 c.p.s.). Thus the apparatus in its present form is suitable for the detection of a rapid density change of roughly 1×10⁻⁵ when the current in each photo-tube is 0.02 μa, a current increment of 2×10⁻¹² amp. This performance is about fifteen times better than that of the apparatus described previously — see Fig. 4 of that paper. A part of this improvement is certainly due to a more accurate adjustment of the band width to suit the speed of the chemical reaction as shown by comparing the nature of the noise on the two figures. The remainder of the improvement is probably due to a higher-gain light-control circuit.

From independent spectrophotometric calibrations.

2. SINGLE-ENDED PHOTO-CELL CIRCUIT

2.1 Design

A similar measurement and control circuit was constructed for a grating monochromator and the circuit is given in Fig. 9. Here a single photo-tube is used for measurement in place of the differential circuits shown previously, and bucking-out voltage is included so that the amplifier usually operates as a null indicator. The bucking-out voltage is suitably switched for substitution spectrophotometry; dark, solvent, and solvent plus solute. In this case rapid changes of light intensity need not be recorded.

Coleman Electric Company.
and the input is therefore bypassed with 0.1-uf condensers. Equal grid resistors are also used here to avoid measuring the drift of grid current of the amplifier. The light-control circuit uses a single photo-cell provided with a blue filter. The output voltage is amplified and controls a type 6AS7G and thereby regulates both the light intensity and the heater voltage for all the amplifiers.

A photograph of the unit complete except for power supply and voltage-dropping resistors is shown in Fig. 10 and includes the double monochromator, cuvette-holder, and modulator, demodulator, and control circuits. In addition, a regulated high voltage supply for a photomultiplier is provided (see Section 3). The cuvette holder is readily replaced by the rapid flow apparatus so that the spectra of unstable intermediate compounds may be obtained.

2.2 Performance

The performance of the unit is given in Fig. 11, and in this case the "error signal" of the null indicator is shown. The drift is recorded on two time scales at a sensitivity of 46 \( \mu V / \text{div} \) as seen from the 600-\( \mu V \) calibration. On the slow paper speed the drift is 12 \( \mu V / \text{min} \) over a 30-minute interval and, on the fast speed, roughly 15 \( \mu V / \text{min} \) over a 3-minute interval. The corresponding fractional changes are 26 and 32\( \times 10^{-4} \) part/min., since the signal voltage is 465 \( \mu V \) or 0.21 \( \mu A \) (the spectral interval is roughly 7 \( \mu m \)). The recording of a small change of light absorption is shown in Fig. 12 where the gain setting is 300 \( \mu V / \text{div} \) and the signal is 1.5 \( \mu V \) or 3\( \times 10^{-3} \). Here a density change of 1\( \times 10^{-3} \) (2\( \times 10^{-19} a \)) is readily detected at a photo-current of 0.21 \( \mu A \). An increase of the gain of the light-control circuit has improved the performance by a factor of nearly 10. Results obtained with this apparatus are given in more detail.7

3. PHOTO-MULTIPLIER

3.1 Design

The photo-multiplier, which excels in the detection of a small light intensity, is not so satisfactory for the detection of a small change in a moderately large light intensity. At moderate currents (\( \sim 50 \mu A \)) these tubes (type 931) show a fatigue or drift which may be due to a variation of the secondary-emission properties of the multiplier. However, it is desirable to determine the stability of this type of tube in spectral range.1

Fig. 8. Measurement of a rapid reaction in a dilute solution. A 1\( \times 10^{-3} M \) Fe/L hematin enzyme, peroxidase, reacting with 1\( \times 10^{-3} M/L \) cyanide, pH = 4.0, is recorded using a 1-mm circular trough. Corning filter 597 and Wratten filter A-2 plus Corning filter 511½ were used. In Fig. 8a the sensitivity was 1.2 \( \mu V / \text{div} \), and the time scale was 4 sec per division. The density change was roughly 2\( \times 10^{-4} \). Figure 8b shows the reaction of Fig. 8a recorded on a mirror oscillograph. The time markers are 0.5 sec. In addition, the displacement and rate of the fluid through the 1-mm optical trough are indicated in the additional traces.

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regions where the emissivity of the tungsten filament or the photo-sensitivity is small.

A circuit for employing type 931-A in the apparatus of Figs. 9 and 10 is shown in Fig. 13. The light-control and measurement circuits are similar to those used before, except that the latter circuit has considerably less gain and requires only one amplifier unit since signals of several volts are available. On the other hand, the stabilization of the supply voltage for the multiplier requires many components. A 1-kc/sec. audio oscillator using a 6V6GT is supplied with regulated heater and plate supplies (only transients need be removed). The a.c. output voltage is rectified by a selenium rectifier and filtered. An adjustable portion of the rectified output voltage is equated to the regulated 150-v supply at the input terminals of a d.c. amplifier. The output of the latter adjusts the screen voltage of the oscillator. To avoid alterations in the regulated output with changes in photo-multiplier current, the dynodes are supplied from a separate voltage divider and the anode is supplied from 150 v. The voltage divider is, however, not suitable for currents in excess of 50 µA. With the voltage regulator previously described the effect of a 10 percent variation of line voltage is considerably less than $1 \times 10^{-4}$ of the output signal.

### 3.2 Performance

Tests of the drift of the output at various photo-currents, output currents, and gains are
figure 11. Drift of circuit of Fig. 9. Sensitivity is 46 μV/min, and two time scales are used; first 1 div./hr. and then 1 div./min. A calibration of 600 μV is indicated by the rectangular pulse near the end of the record. The drift is roughly 3 × 10⁻⁵ part/min.

shown in Figs. 14 and 15. These experiments were carried out to determine the best that could be expected from a selected type 931-A which had been stabilized for several hours and which was operated at relatively low voltages (gains of several thousand). Other tubes were much less stable.

The tube was then operated at an output current at which fatigue was observable as noted by the rapid decrease of output current shown.

drift resumes at roughly 5 × 10⁻³ per minute. The effect of varying the gain of the multiplier is shown in Fig. 15 where the drift rate at constant output current but at several-fold different light intensity and gain is shown in Runs No. 10 and No. 11 where the drift is roughly the same (4 × 10⁻⁴ per minute) in the last two minutes of the runs. (The early portion of No. 10 is obscured by a recovery phenomenon.) On increasing the output current the drift again increases to 4 × 10⁻³ per minute as shown in Run No. 12. On decreasing the gain and increasing the photo-current at constant output, the drift apparently increases in Run No. 13; this effect is, however, not definitely confirmed.

In these experiments the light-control and measurement circuits were the same as in Fig. 11, where drifts of 0.3 × 10⁻⁴ per minute were obtained, indicating that the effects of Figs. 14 and 15 were probably not due to measurement errors. Furthermore, precautions were taken in the latter experiments to avoid exposure to a light intensity greater than that used in the measurements. These experiments were not carried out at smaller currents, and it is not known whether the drift decreased further with decreasing tube current. But soon the output

density change occurring in the reaction of 1.3 × 10⁻⁷ M Fe/L azide-catalase and 0.8 × 10⁻⁸ M/L H₂O₂ at pH = 7.0 (Azide = 40 × 10⁻⁶ M/L) at a wave-length of 400 mμ and a spectral interval of 7 mμ. The sensitivity is 300 μV/div. and the time scale is 1 div./min. The total density change is 1 × 10⁻³. The abrupt downward deflection at the beginning of the record marks the moment at which H₂O₂ was added to the solution. The compound spontaneously decomposes in about a minute.

In Run No. 6, Fig. 14, the drift is 8 × 10⁻³ per minute. A reduction of light intensity by ½ (by changing the wave-length setting of the monochromator) results in a drift of 5 × 10⁻⁴ per minute as shown in Run No. 7. On again increasing the output current at two different wave-lengths (Runs No. 8 and No. 9), the rapid
drift resumes at roughly 5 × 10⁻³ per minute. The effect of varying the gain of the multiplier is shown in Fig. 15 where the drift rate at constant output current but at several-fold different light intensity and gain is shown in Runs No. 10 and No. 11 where the drift is roughly the same (4 × 10⁻⁴ per minute) in the last two minutes of the runs. (The early portion of No. 10 is obscured by a recovery phenomenon.) On increasing the output current the drift again increases to 4 × 10⁻³ per minute as shown in Run No. 12. On decreasing the gain and increasing the photo-current at constant output, the drift apparently increases in Run No. 13; this effect is, however, not definitely confirmed.

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FIG. 14. Drift of a photo-multiplier. The various tests indicate the effect of varying output current and wavelength. The increased stability at lower output currents is shown.

current of the multiplier approaches that obtainable from the ordinary photo-tube as used in this monochromater (0.2 $\mu A$), and the multiplier affords no advantage at all. The stability of

this particular multiplier ($\sim 4 \times 10^{-4}$ parts per minute) does not approach that of the phototube ($\sim 3 \times 10^{-5}$ parts per minute).

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